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Schizophyllan (SPG)-treated macrophages and anti-tumor activities against syngeneic and allogeneic tumor cells

I. Characteristics of SPG-treated macrophages

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Summary. *We tested anti-tumor activities of macrophages treated with a neutral polysaccharide, schizophyllan (SPG), against syngeneic and allogeneic tumor cell lines. SPG was a macrophage stimulant which was not mitogenic to lymphocytes. That made a sharp contrast with the data that Corynebacterium parvum, BCG, and muramyl dipeptide (MDF) were macrophage stimulants which had lymphocyte-activating properties. Treatment of SPG-treated PEC with Thy12 monoclonal antibody and guinea pig complement did not affect the capabilities of tumor-cell-growth suppression by the treated PEC. Thus, the effector cells were peritoneal adherent cells (macrophages morphologically) and effector-to-target contact seemed to be necessary for effective tumor-cell-growth inhibition, although contradictory data exist for this. Murine peritoneal adherent cells harvested 4 days after a single IP injection of SPG at a dose of 100 mg/kg body weight of mouse showed the most prominent cytostatic and cytotoxic activities against syngeneic and allogeneic tumor cells. The distribution of anti-tumor activity in macrophages of various sizes followed the same pattern as macrophages treated with C. Parvum, i.e., larger macrophages showed more remarkable anti-tumor activity. Crude nonadherent peritoneal cells incubated with SPG at a concentration of 10 μg/ml, 100 μg/ml, or 1 mg/ml did not secrete lymphokine that rendered macrophages cytotoxic, while ConA-treated nonadherent cells did so. Furthermore, spleen cells treated with SPG in vivo did not secrete macrophage-activating lymphokine in the presence of SPG. On the other hand, addition of 1 mg/ml of SPG-treated peritoneal adherent cells and bone-marrow-derived macrophages in vitro rendered them cytotoxic to a moderate degree. This implies that SPG may activate macrophages directly, allowing them to become cytotoxic in the peritoneal cavity. Lastly, SPG could induce production of Il-1-like factor to a moderate degree. SPG, whose molecular structure is well elucidated, will provide us with a strong tool to analyze the mechanism of macrophage activation both in vitro and in vivo.*

Introduction

Schizophyllan (SPG) is a glucan extracted from the culture filtrate of Schizophyllum commune Fries. It consists of repeating units composed of three or four β-(1→3)-linked D-glucopyranose residues, to one of which is attached, through β-(1→6)-linkage, a side chain consisting of a single β-D-glucopyranose residue [11].

The anti-tumor activity of SPG was first observed on transplanted murine tumors [11] and Lewis lung carcinoma as a tumor metastasis model [33] and later on rat tumors as well [22]. Furthermore, T-cell adjuvant activity has also been reported [10].

Very recently, it has been suggested that T cells and macrophages are responsible for the anti-tumor activity in SPG-treated mice because it was diminished after treatment with anti-Thy1.2 and complement and in vivo treatment of carrageenan or trypan blue [31]. However, the mechanism of any macrophage to T-cell interaction and macrophage cytotoxicity induced by SPG remains obscure.

Thus, we performed experiments, aiming at defining the role of peritoneal nonadherent cells (mostly lymphocytes) in the development of anti-tumor activity and the effects of macrophage subpopulations on murine syngeneic and allogeneic tumor cell lines.

We found that the anti-tumor activity resides mainly in the largest macrophages. The data suggest that nonadherent cells (containing lymphocytes) do not play an important role in activating peritoneal macrophages and tumor cell growth inhibition and that at higher concentration SPG activates macrophages directly rather than through T cells.

Materials and methods

Mice. DBA/2 male mice, 6–8 weeks old, were purchased from the Jackson Laboratory, Bar Harbor, Maine or were bred locally by the Health Sciences Animal Centre, University of Alberta.

Chemicals. Schizophyllan (SPG) (pyrogen free, Mr $4-5 \times 10^5$) was kindly provided by Kaken Chemical Co., Tokyo, Japan, and dissolved in saline. KAC-2 (silica suspension) was obtained from Nippon Kotai kenkyujo, Gunma, Japan.

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Abbreviations: PEC, peritoneal exudate cells; SPG, schizophyllan; LPS, lipopolysaccharide; Con A, concanavalin A; CGN, carrageenan; B. M., bone marrow; FCS, fetal calf serum; BCG, bacille Calmétte Guérin; Il-1, interleukin 1; PPD, pure protein derivatives; MDP, muramyl dipeptide; C. parvum, Corynebacerium parvum

Lambda carrageenan (CGN) was purchased from Sigma Chemicals Co., St. Louis, Mo., USA.

Tumor cell lines. LY59F9 (leukemia line) and SL2R5 (lymphoma line), both derived from DBA/2 mice, and Yac/R11 (lymphoma line), derived from A/Sn mice, were kindly supplied by Dr. A. Greenberg, Mannitoba Institute of Cell Biology, University of Mannitoba, Winnipeg. They were maintained in vitro in RPMI 1640 and 10% fetal calf serum and transferred every 4 days. The lines were used at exponential phase.

Preparation of peritoneal exudate cells (PEC) and fractionation of PEC by velocity sedimentation at unit gravity. PEC were harvested from mice by lavage of the peritoneal cavity with Puck's saline A and were irradiated with 2,000 rads of r-radiation from a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd., Ottawa, Ontario) prior to use to inactivate non-macrophage cells. Stimulated PEC were obtained by a single IP injection of SPG at a dose of 10, 50, 100, or 200 mg/kg body weight and harvested 0, 4, 7, and 10 days later.

Separation of cells on the basis of size by unit gravity velocity sedimentation was performed at 4° C in a Sta-put apparatus (17.5 cm in diameter) as described by Miller and Phillips [13, 21]. Cell recovery was always greater than 70%.

Assay for suppression of tumor cell growth. The methods were the same as those used by Lee and Berry [13], who utilized the uptake of tritiated thymidine as a measure of tumor cell growth. Measured numbers of PEC or cell fraction A to E were incubated in triplicate in flat-bottomed microculture plates (no. 3040, Falcon Plastics, Oxnard, Calif.). Thereafter, tumor target cells (10^4) were placed in each well. Each well contained 0.2 ml of RPMI 1640 supplemented with glutamine, streptomycin-penicillin and 10% fetal calf serum (FCS). After 2 days' incubation, 20 µl of RPMI 1640 containing [methyl-^3H]-thymidine was placed into each well and pulsed for additional 6 h (25 µCi/ml, 2 Ci/mmol; New England Nuclear, Boston, Mass.). The cells in the wells were harvested onto glass fiber filters with Titertek cell harvester (Flow Laboratories). The filters were dried and the radioactivity was determined by liquid scintillation counting.

In some experiments, silica (25 µg/ml) and λ-carrageenan (CGN) (500 µg/ml), both macrophage-toxic agents, were used to demonstrate that the effector cells were phagocytic cells [3, 6, 29]. Peritoneal adherent cells were incubated with either silica or λ-CGN at 37° C for 4 h. Thereafter, the medium was replaced and appropriate numbers of tumor target cells added.

In addition, in some experiments, SPG-treated PEC were treated with Thy12 monoclonal antibody (NEI001, New England Nuclear) and guinea pig complement [30]. Briefly, 1 : 750 diluted Thy12 was added to 10^7/ml SPG-treated PEC and the cells were incubated at 4° C for 45 min. Thereafter, 1 : 5 diluted guinea pig complement was added and incubation was carried out further at 37° C for 45 min. After washing twice in RPMI 1640, the cells were placed into microculture plates.

In other experiments, Marbrook's culture bottles (no. 7, Takahashi Giken Co., Tokyo, Japan) were used to investigate if effector-to-target cell contact was necessary for suppression of tumor cell growth and humoral factor release by adherent cells. In this system, the SPG-treated macrophages (10^7 cells in 1 ml in the inner bottle) were separated from the target tumor cells in the outer bottle (5×10^5 in 10 ml) by a Millipore membrane filter (0.45 µm in diameter). Incubation was carried out for 2 days. The cell suspension (0.2 ml) in the outer bottle was transferred to microculture wells, pulsed with [methyl-^3H]-thymidine for 6 h, harvested and counted. As controls, PEC and tumor cells or tumor cells alone was incubated in the outer tube.

Assay for killing of tumor cells. This assay was performed under culture conditions identical to those for the suppression of tumor cell growth, with the exception that 10^6 in 1 ml tumor target cells had been labelled with 1 µCi/ml of ^{125}I-iodo-deoxy-uridine (IUdR) for 4 h [14]. After 2 days of culture, 0.1 ml of the supernatant was aspirated off for the determination of ^{125}I release by a gamma counter. The percentage of specific lysis is defined as:

$$\frac{\text{Experimental counts-spontaneous release}}{\text{Maximal release-spontaneous release}} \times 100\,(\%)\,.$$

Differential cell counts for morphological classification. Cell preparations were prepared by the Cytospin centrifuge (Shandon Southern Instruments Inc., USA) and stained with Giemsa stain for morphological classification [5].

Preparation of lymphokine-containing supernatants from peritoneal exudate lymphocytes treated with SPG in vitro and spleen cells treated with SPG in vivo; evaluation of the capacity of the supernatants to render peritoneal adherent cells cytotoxic. To investigate whether SPG can induce peritoneal exudate lymphocytes to secrete macophage-activating lymphokine PEC from unstimulated peritoneal cavity were incubated on plastic petri dishes at 37° C for 1 h and the crude nonadherent cells obtained. After washing twice, 2×10^6 crude nonadherent cells that still contained adherent cells were incubated in the presence of SPG (10 µg/ml, 100 µg/ml or 1 mg/ml) at 37° C in a CO_2 incubator for 24 h. Thereafter, the cell suspensions were centrifuged at 3,000 rpm for 30 min at 4° C, and the supernatants were collected, filtered and immediately used. Positive control lymphokine-containing supernatants were induced by Con A (2 µg/ml).

Lymphokine-containing supernatants were also prepared by a different method. Briefly, mice were injected IM with 10 mg/kg body weight SPG. We adopted this dosage because macrophages obtained after the injection showed the most prominent anti-tumor activity (unpublished observations and [31]). Seven days after injection the spleens were excised. After removal of erythrocytes by centrifugation over Ficoll-Isopaque, 5×10^6 spleen cells were incubated for 2 days at 37° C with or without SPG at 10 µg/ml, 100 µg/ml or 1 mg/ml. After spinning down at 2,500 rpm for 15 min, the supernatants were collected, filtered, and immediately used.

To evaluate the capacity of the supernatants to render peritoneal adherent cells cytotoxic, unstimulated, irradiated adherent cells were cultured with the supernatants and/or SPG (1 mg/ml) for 8 h at 37° C at a final concentration of 1 : 4 or 1 : 8 [28]. Thereafter, the medium was replaced and 10^4 tumor cells added to each well. Incubation was further carried out for 2 days, after which 20 µl of [methyl-^3H]-thymidine-containing solution was administered for assay of tumor cell survival.

Preparation of bone-marrow-derived macrophages. Mouse femur and tibia were removed aseptically and the bone marrow

plugs were flushed out with Puck's saline A. After washing twice, 1×10^6 bone marrow cells in 25 ml were cultured for 5 days on petri dishes in medium supplemented with 10% FCS, 10% horse serum, and 10% L-cell-derived conditioned medium as a source of macrophage growth factor [16]. The adherent cells were recovered with use of PBS-EDTA solution, washed twice and suspended in RPMI 1640 medium. The bone-marrow-cultured adherent cells contained more than 98% macrophages, consistent with morphological criteria of macrophages [5]. To ascertain whether SPG can activate bone marrow macrophages in vitro to suppress tumor cell growth, a fixed number of macrophages (10^5, 5×10^4 or 2.5×10^4/microwell) was placed into microwells with 1 mg/ml SPG and cultured for 6 h. Thereafter, 10^4 tumor target cells were added to assay suppression of tumor cell growth as described previously.

Blastic transformation test. Spleen cells and thymus cells were obtained to investigate if SPG was a lymphocyte mitogen. Briefly, spleen cells (5×10^5/well) or thymus cells (1×10^6/well) were placed into microculture plates with various concentrations of SPG (2 mg/ml, 1 mg/ml, 500 μg/ml, 100 μg/ml, 50 μg/ml or 10 μg/ml) and cultured for 2, 3, and 7 days. Thereafter, the cells were pulsed with [methyl-^{3}H]-thymidine, harvested, and counted as usual. The system for study of antigen-specific T-cell proliferation was also used [15]. Mice were treated in the footpad with SPG (400 μg/mouse) in Freund's complete adjuvant. Eight days later, popliteal lymph node cells were cultured in microculture plates with SPG (1 mg/ml) or PPD (100 μg/ml). Four days later, the degree of antigen-specific T-cell proliferation was evaluated in terms of ^{3}H-thymidine uptake.

Production of Il-1-like humoral factor by SPG and assay for Il-1 like activity. Crude adherent peritoneal cells were utilized to obtain Il-1-containing supernatants. Briefly, 2×10^6/ml crude adherent peritoneal cells were cultured in 2 ml in 12-well culture plates with SPG (1 mg/ml) or LPS (10 μg/ml) for 2 days. Thereafter, the culture supernatants were collected, filtered, and immediately tested for Il-1-like activity. Il-1 assay was performed as stated by Mizel [23]. The supernatant at a dilution of 1:8 was added to 10^6C3H/HeJ thymocytes with or without Con A (1 μg/ml), and the cell suspensions were cultured in a flat-bottomed microculture plate for 3 days at 37° C in a CO_2 incubator. Il-1-like activity was evaluated in terms of ^{3}H-thymidine uptake.

Results

Cellular analysis of velocity sedimentation at unit gravity of SPG-treated PEC and cytostatic and cytotoxic activities of SPG-treated PEC and fractionated PEC against various tumor cell lines

Maximal cytostatic activity against syngeneic and allogeneic tumor cells was manifested by peritoneal exudate cells 4 days after a single IP injection of SPG (Fig. 1). The cytostatic activity correlated well with cytotoxic activity when assayed against a variety of tumor cell targets (Fig. 2). SPG-treated peritoneal nonadherent cells did not kill tumor cells in vitro at any effector-to-target ratio.

Table 1 shows the cell profile of the collected cell fractions. The cells isolated from the high-velocity cell fractions (fractions D and E) were predominantly macrophages. On the other hand, the low-velocity cell fractions (< 5.0 mm/h) contained few macrophages. These data have the same pattern as *C. parcum*-treated PEC fractionation [13, 14].

Unit gravity velocity sedimentation separation of the SPG-treated PEC revealed that the anti-tumor function was

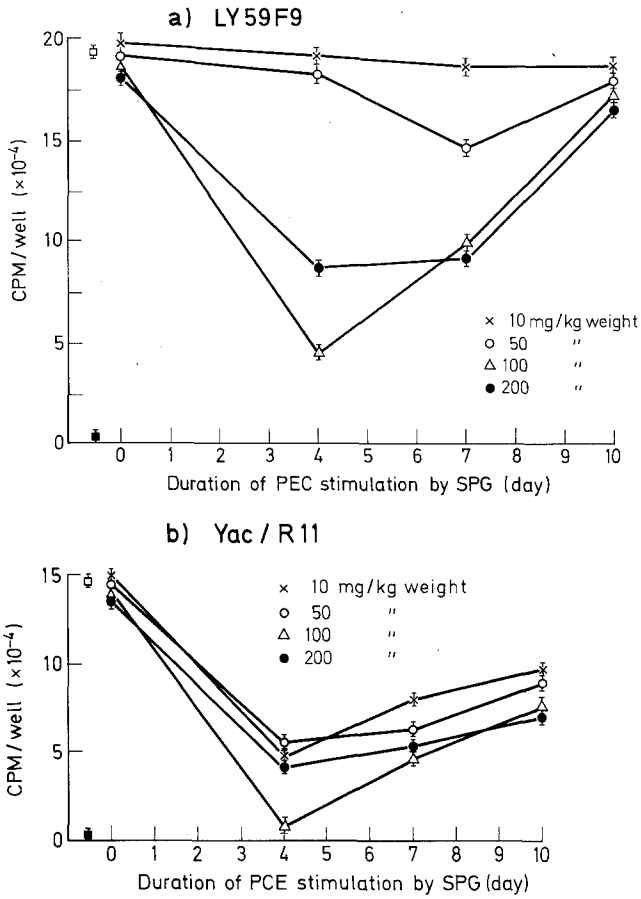

Fig. 1. Time course of anti-tumor cytostatic activity after a single IP injection of SPG at various doses. PEC harvested after various times after a single IP injection of SPG (10–200 mg/kg body weight) were tested for the ability to inhibit [methyl-^{3}H]-thymidine uptake by various tumor cells. Effector to tumor target ratio is 20:1. ■; irradiated PEC alone, □; tumor cells alone. The *vertical bars* represent standard deviations

Table 1. Composition of SPG-treated PEC, fractionated by unit gravity velocity sedimentation[a]

Fraction (sedimentation velocity in mm/h)	% Recovered cells[b]	% Cells in fraction[c]		
		Monocyte/ macrophage	Lymphocyte	Others
A (3.3–0.0)	15	9.8	87.4	2.8
B (5.0–3.3)	35	25.0	71.0	4.0
C (7.0–5.0)	28	81.3	16.3	2.4
D (9.2–7.0)	17	90.4	8.9	0.7
E (14.7–9.2)	5	98.0	1.0	1.0
Unfractionated		61.0	35.4	3.6

[a] 4-day SPG-treated PEC were fractionated by velocity sedimentation technique. Specimens of the pooled fraction A–E were prepared with cytospin centrifuge and stained with Giemsa stain for morphological classification

[b] 71% of the initial cells recovered after fractionation

[c] At least 500 cells screened

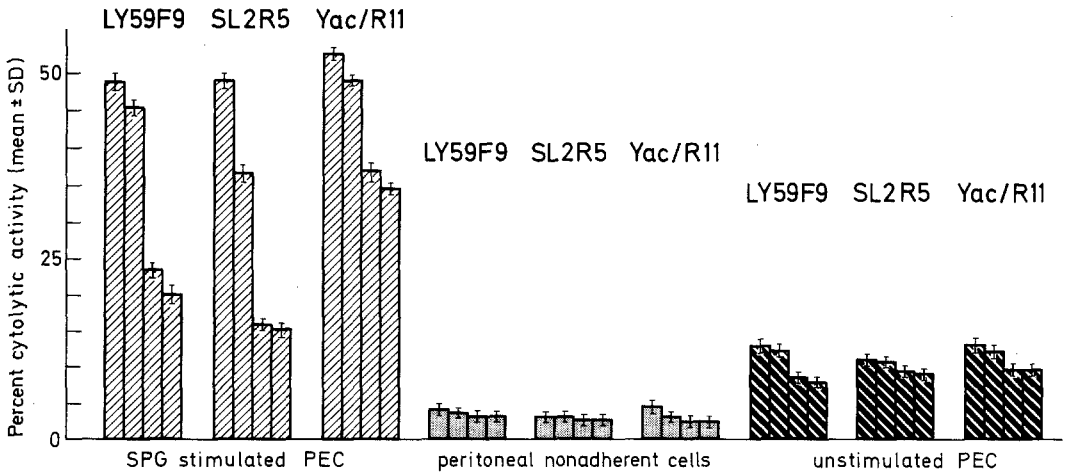

Fig. 2. Killing of various tumor cells by SPG-treated PEC. PEC were collected 4 days after a single IP injection of 100 mg/kg weight of SPG. Various numbers of irradiated SPG-treated PEC, SPG-treated peritoneal nonadherent cells and irradiated unstimulated PEC were added to 10^4 ^{125}IUdR-labelled tumor cells; 2 days later, 0.1 ml of the supernatants was counted in a gamma counter. *From the left*, effector to target cell ratio is 20 : 1, 10 : 1, 5 : 1, and 2.5 : 1, respectively. The *vertical bars* show standard deviations

Table 2. Effect of lambda carrageenan-, silica-, and Thy-12-treated SPG and separately cultured SPG-treated PEC on anti-tumor cytostatic activity[a]

	PEC	Treatment of PEC	Tumor targets ($\times 10^{-3}$)		
			LY 59F9	SL2R5	Yac/R11
Expt. 1	−	−	95.5 ± 6.8	208.7 ± 6.0	73.3 ± 7.9
	+	+	11.0 ± 1.8	20.3 ± 1.0	1.3
	−	Lambda CGN (500 μg/ml)	75.6 ± 3.1	177.6 ± 9.1	60.3 ± 3.7
	+	Lambda CGN (500 μg/ml)	82.1 ± 6.7	185.1 ± 2.1	69.7 ± 1.5
	−	Silicia (25 μg/well)	72.0 ± 3.8	145.2 ± 2.0	57.7 ± 1.0
	+	Silicia (25 μg/well)	77.1 ± 2.1	146.6 ± 5.8	64.3 ± 3.5
Expt. 2	+	−		26.7 ± 4	
	+	Thy 12 +c′[b]		10.4 ± 1	
	+	Thy 12		25.4 ± 1.6	
	+	c′		28.8 ± 3	
	−	−		58.1 ± 0.8	
Expt. 3	PEC (cocultured with tumor cells in Marbrook's culture bottle)[c]		0.2	2.4	0.3
	PEC and tumor cells (separately cultured)[d]		11.9 ± 0.9	20.1	13.1 ± 0.8
	Tumor cells alone cultured in an outer bottle of Marbrook's culture bottle with medium in an inner tube		15.7 ± 0.9	19.6 ± 0.5	14.1

[a] 2×10^5 SPG-treated irradiated PEC were treated with lambda CGN (500 μg/ml) or silica (KAC-2 25 μg/well) for 4 h and washed off once. Thereafter, 10^4 tumor cells were added, cultured for 2 days, pulsed with [^{3}H-methyl]-thymidine for 6 h, harvested and counted. Each value shows mean ± SD (cpm). SD values < 500 not shown

[b] PEC : tumor target = 10 : 1. The count of 10^5 PEC alone was 1,145 ± 52. The count of the spleen cells (5×10^5/well) treated with Thy 12 + c′ was 1,270 ± 27 in the presence of Con A (2 μg/ml). The count of spleen cells in the presence of Con A was 117,738 ± 10,065

[c] 5×10^6 SPG-treated irradiated PEC in 1 ml and 2.5×10^5 tumor cells in 10 ml were cocultured in outer bottles of Marbrook's culture bottles for 2 days and thereafter, 200 μl of the cell suspensions was transferred to microculture plates, pulsed for 6 h and counted

[d] 5×10^6 SPG-treated (irradiated) PEC in 1 ml were cultured in inner tubes sequestered by non-toxic Millipore filter and 2.5×10^5 tumor cells in 10 ml were cultured in outer bottles of Marbrook's culture bottles for 2 days and followed c

mainly associated with large cells (Fig. 3) which consisted mainly of macrophages (Table 1).

Effect of lambda carrageenan, silica, Thy12, and complement as well as sequestered incubation of SPG-treated PEC and tumor cells in Marbrook's culture bottles, on anti-tumor activity

The nature of the anti-tumor effector cells was further characterized by agents such as carrageenan (CGN) and silica which were toxic to phagocytic cells. Table 2 shows that SPG-treated PEC treated with lambda CGN and silica did not have cytostatic activity against various tumor cell lines, whereas PEC as a control had normal activity. Treatment of SPG-treated PEC with Thy12 and guinea pig complement did not affect the capability of the treated PEC to suppress tumor cell growth. We also tested whether SPG-treated PEC can affect tumor cells without cellular contact, using Marbrook's culture bottles. Table 2 reveals that effector-to-target cell contact seems to be necessary. Thus, SPG-treated PEC did not affect tumor cells across a Millipore membrane filter.

Table 3. Effect of supernatants from Con-A- or SPG-treated peritoneal nonadherent cells on anti-tumor cytostatic activity[a]

PEC	Source of supernatant	Addition of SPG (1 mg/ml)	Tumor targets ($\times 10^{-3}$)		
			LY59F9	SL2R5	Yac/R11
+	Con A	−	50.7	105.2 ± 1.2	44.2 ± 1.6
+	Con A	+	19.3 ± 1.5	34.9 ± 9.0	13.1 ± 1.6
+	SPG	−	80.5 ± 5.7	189.5 ± 1.7	65.4 ± 1.8
+	SPG	+	31.3	39.9 ± 0.9	17.0 ± 2.8
+	−	+	31.2 ± 2.4	45.9 ± 1.3	18.1 ± 1.8
+	−	−	91.1	195.2 ± 1.2	64.7 ± 2.0
−	−	−	86.0 ± 5.5	215.5 ± 9.3	74.9 ± 1.3
+	−	−	(586 ± 29)[b]	(434 ± 12)	(389 ± 83)
−	Con A	−	93.9 ± 7.9	207.1 ± 7.7	66.7 ± 1.7
−	SPG	−	90.9	223.9 ± 3.5	70.9 ± 0.9
−	−	+	89.7	213.0 ± 1.6	76.1 ± 6.7
−	Cells alone	−	84.3 ± 2.0	193.8 ± 12.3	76.4 ± 8.1
+	Cells alone	+	35.3 ± 1.1	43.8 ± 1.4	19.3 ± 0.8
+	Cells alone	−	86.9 ± 5.6	203.4 ± 7.6	71.8 ± 4.3

[a] The supernatants from Con-A- or SPG-treated crude peritoneal nonadherent cells were added at a final dilution of 1 : 8 to 2 × 10^5 unstimulated and irradiated PEC and incubation was carried out for 10 h. Thereafter, 10^4 various tumor cells were placed and incubation was further carried out for 2 days. Each combination was pulsed with [methyl-^{3}H]-thymidine for 6 h, harvested and counted. Each count shows mean ± SD (cpm), each in triplicate. SD values < 500 not shown

[b] The values in parenthesis refer to PEC without addition of tumor cells

Table 4. Effect of supernatants from in vivo SPG-treated spleen cells on anti-tumor cytostatic activity[a]

PEC	Source of supernatant	Addition of SPG	Tumor targets ($\times 10^{-3}$)		
			LY59F9	SL2R5	Yac/R11
+	−	−	101.1 ± 1	94.3 ± 1.7	34.3 ± 1.8
+	−	+	58.4 ± 1.1	23.2 ± 0.8	7.6
+	Cells alone	−	90.3 ± 1.1	96.1 ± 1.0	33.6
+	Cells alone	+	56.3 ± 2.2	17.8	5.2
+	SPG (1 mg/ml)	−	114.0 ± 3.7	83.6 ± 3.4	29.8 ± 0.8
+	SPG (1 mg/ml)	+	62.3	23.8 ± 6.0	7.9
+	SPG (100 μg/ml)	−	112.2 ± 8.3	84.2 ± 5.3	29.7 ± 1.4
+	SPG (100 μg/ml)	+	51.7 ± 1.5	39.0 ± 1.2	7.0 ± 1.1
+	SPG (10 μg/ml)	−	119.6 ± 1.0	82.0 ± 1.2	26.9 ± 1.4
+	SPG (10 μg/ml)	+	30.7 ± 2.8	34.1 ± 4.3	6.6 ± 0.6
−	−	+	116.5 ± 5.4	108.7 ± 5.8	51.4 ± 0.5
−	−	−	121.6 ± 1.6	100.1 ± 5.4	56.7 ± 2.0
−	SPG (1 mg/ml)	−	136.2 ± 3.6	124.8 ± 3.8	48.5 ± 1.1

[a] First, mice were treated with intramuscular injection of SPG (10 mg/kg body weight). Seven days later 5 × 10^6 spleen cells were incubated for 48 h at 37° C with or without addition of SPG (1 mg/ml, 100 μg/ml, or 10 μg/ml). The supernatants from various sources were tested at a final dilution of 1 : 4 for their capacity to render the irradiated and unstimulated PEC cytotoxic. All the values show mean ± SD (cpm), each in triplicate. SD values < 500 not shown. The count of PEC alone was less than 600 cpm

Effect of the supernatants from in vitro SPG, Con-A treated peritoneal nonadherent cells, or spleen cells treated with SPG in vivo on anti-tumor cytostatic activity

As depicted in Table 3, the supernatants obtained from Con-A treated peritoneal nonadherent cells induced cytostatic activity in unstimulated PEC, whereas the supernatants from SPG-treated peritoneal nonadherent cells did not. Very interestingly, when SPG at a high concentration (1 mg/ml) was added to unstimulated, irradiated PEC and tumor cells, tumor cell growth suppression was recognized as significant, and at that dose SPG was not toxic to tumor cells. The presence of both SPG and Con-A supernatant produced an enhancing anti-tumor effect.

The data in Table 4 show that supernatants from in vivo SPG-treated spleen cells in the presence of SPG produced the same effect as the supernatants from cells treated with SPG in vitro. Thus, addition of SPG (1 mg/ml) augmented anti-tumor cytostatic activity by irradiated PEC, but supernatants from any sources did not enhance the tumor cell suppression activity by irradiated PEC.

Cytostatic activity of in vitro SPG-treated bone-marrow-derived macrophages against various tumor cells

Highly purified macrophages derived from bone marrow were utilized to confirm whether an appropriate dose of SPG can activate the macrophages in vitro to suppress tumor cell growth. Macrophages derived from in vitro SPG-stimulated bone marrow inhibited the tumor cell growth significantly, but

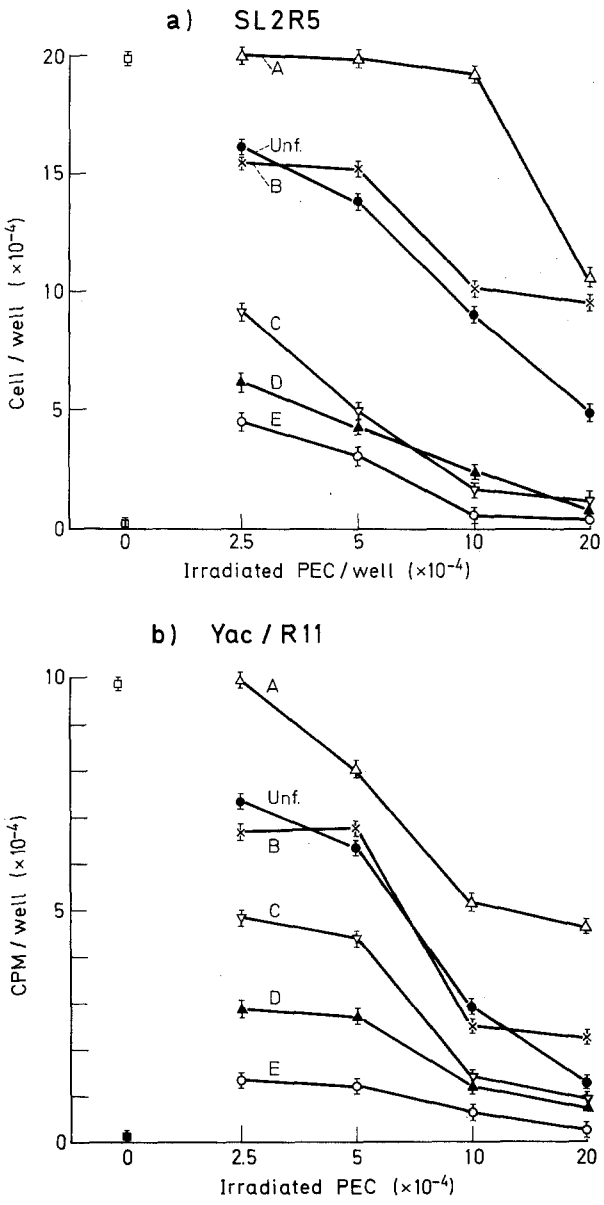

Fig. 3. Cytostatic activities of SPG-treated PEC, fractionated by unit gravity velocity sedimentation. Unfractionated PEC and fractions A–E were tested for inhibition of the [methyl-^{3}H-]thymidine uptake of 10^4 various tumor cells: *A*, fraction A; *Unf.*, unfractionated PEC. The *vertical bars* represent standard deviations. ■; count of PEC alone, □; count of tumor cells alone

bone marrow derived macrophages without SPG displayed considerable tumor-static activity (Table 5).

Blastic transformation test

As shown in Table 6, SPG did not induce mitogenic responses to murine spleen cells and thymus cells at five different concentrations. Furthermore, we performed experiments on the proliferation of SPG-pretreated lymph-node cells in the presence of SPG, but the data were not significant (Table 6).

Assay for evaluating the Il-1-like humoral factor

We assessed the activity of the I1-1-like factor from peritoneal cells treated with SPG in vitro. The supernatant from

Table 5. Cytotoxic activity of in vitro SPG-treated bone marrow derived macrophages against tumor cells[a]

Number of bone marrow macrophages	Addition of SPG (1 mg/ml)	Tumour targets ($\times 10^{-3}$)	
		LY59F9	SL2R5
10^5	–	60.1 ± 1.4	2.4
5×10^4	–	169.4 ± 14.7	8.2
2.5×10^4	–	175.4 ± 3.5	8.3 ± 0.5
10^5	+	36.8 ± 2.3	1.2
5×10^4	+	106.0 ± 7.7	5.1
2.5×10^4	+	133.7 ± 6.9	4.0
–	+	177.3 ± 1.2	10.1
–	–	177.4 ± 1.1	11.5
10^5	–	(802 ± 21)[b]	(480 ± 75)

[a] Five-day-cultured bone-marrow-derived macrophages were placed into microculture plates and cultured with 1 mg/ml SPG for 6 h. Thereafter, 10^4 tumor cells were added, cultured for 2 days, pulsed, harvested and counted. All the values show mean ± SD (cpm). SD values < 500 left out

[b] The values in parentheses refer to bone-marrow-derived macrophages without addition of tumor targets

SPG-treated peritoneal cells showed considerable I1-1-like activity in terms of thymocyte proliferation as compared to that from LPS-treated peritoneal cells in vitro.

Discussion

In this paper we showed that the anti-tumor effector cells in the peritoneal cavity induced by SPG were mainly large macrophages, and cell-to-cell contact seemed to be necessary for effective tumor-cell growth inhibition. SPG appeared to activate peritoneal and bone-marrow-derived macrophages to render them cytotoxic in a direct manner just like LPS [8] and double-stranded RNA [2] (Table 3).

We wanted to elucidate the mechanism of macrophage activation by SPG. We observed that SPG could activate unstimulated and irradiated PEC directly in vitro. This was to some extent confirmed by use of bone-marrow-derived macrophages, which were practically devoid of lymphocyte contamination. Although the macrophage growth factor contained in the L-cell-conditioned medium stimulates macrophages somewhat [20], the additional stimulatory effect of SPG was significant.

Unstimulated PEC consists of lymphocytes, macrophages, polymorphs, and mast cells. Lmphocytes make up 40%–60% of the unstimulated PEC (unpublished observations and [13]). Therefore, it is important to know whether such lymphocytes play any role in peritoneal macrophage activation. Table 2 shows that the activated macrophages can suppress tumor cell growth in vitro at an effector phase without T-cell help. As shown in Table 3, the lymphocytes can respond to an appropriate stimulus (here ConA) to secrete lymphokine, which activates peritoneal, unstimulated macrophages. SPG did not induce lymphokine secretion in vitro and in vivo. However, we cannot say that lymphocytes do not play a role in macrophage activation. Further detailed study is required to elucidate the role of lymphocytes in macrophage activation.

Recently Mizuhira et al. report that SPG is a neutral, nondegradable polysaccharide, and "filamentous balls" consisting of SPG and strong acid phosphatase activity were found

Table 6. The effect of SPG on murine lymphocyte proliferation

Kind of cells added		Addition of a chemical	Count (cpm)			
			Day 2	Day 3	Day 4	Day 7
Expt. 1	s	SPG (2 mg/ml)	1,523 ± 27	2,307 ± 11		3,317 ± 81
	s	SPG (1 mg/ml)	1,723 ± 201	2,413 ± 21		3,617 ± 80
	s	SPG (0.5 mg/ml)	1,693 ± 148	2,511 ± 80		3,518 ± 20
	s	SPG (50 μg/ml)	1,367 ± 226	2,567 ± 91		2,918 ± 31
	s	SPG (5 μg/ml)	1,504 ± 485	2,318 ± 16		3,019 ± 65
	s	–	1,692 ± 130	2,270 ± 13		3,517 ± 14
	th	SPG (2 mg/ml)	200 ± 8	120 ± 40		36 ± 1
	th	SPG (1 mg/ml)	163 ± 3	136 ± 1		43 ± 1
	th	SPG (0.5 mg/ml)	126 ± 14	143 ± 8		80 ± 2
	th	SPG (50 μg/ml)	118 ± 1	160 ± 3		55 ± 4
	th	SPG (5 μg/ml)	111 ± 19	166 ± 14		63 ± 2
	th	–	130 ± 4	109 ± 5		56 ± 6
	th	LPS (10 μg/ml)	129 ± 17	172 ± 8		70 ± 2
	th	Con A (2 μg/ml)	4,813 ± 21	7,646 ± 19		35 ± 1
Expt. 2	LN	SPG (2 mg/ml)			6,523 ± 27	
	LN	SPG (1 mg/ml)			8,849 ± 596	
	LN	SPG (0.5 mg/ml)			6,693 ± 143	
	LN	SPG (50 μg/ml)			6,367 ± 226	
	LN	SPG (5 μg/ml)			6,504 ± 485	
	LN	PPD (100 μg/ml)			76,229 ± 7,847	
	LN	–			6,732 ± 557	

All the values show mean ± SD (cpm), each in triplicate
s = spleen cells, *th* = thymus cells, *LN* = lymph node cells (4×10^5/well)

Table 7. The production of Il-1-like humoral factor[a]

Thymus cells	Source of supernatant	Addition of Con A	1 : 8 dilution of Il-1 added to the cultures (^{3}H-thymidine uptake)
+	Cells alone	–	367 ± 18
+	Cells alone	+	29,257 ± 1,523
+	SPG (1 mg/ml)	–	1,090 ± 100
+	SPG (1 mg/ml)	+	45,518 ± 6,207
+	LPS (10 μg/ml)	–	130 ± 44
+	LPS (10 μg/ml)	+	48,062 ± 5,356

[a] Two milliliter of crude adherent peritoneal cells was cultured in a 24-well cultured plate at a density of 2×10^6/ml in RPMI 1640 containing 5% FCS and stimulated with LPS or SPG. Cultures were incubated at 37° C for 48 h in a CO_2 incubator. The supernatant at a final dilution of 1 : 8 was added to 10^6 C3H/HeJ thymocytes with or without Con A, and the cell suspension was cultured for 72 h at 37° C in a CO_2 incubator. All the counts show mean ± SD (cpm), each in triplicate

in the SPG-treated macrophages at the ultrastructural level [24, 25]. When our data and theirs are taken together, they suggest that SPG-phagocytosing mature macrophages are cytotoxic and SPG may be acting as a direct activator of macrophages in our in vitro system. It is natural that macrophages may be activated by other mechanisms such as T cells and their humoral factor [9, 12, 27] because we could not analyze the role of peritoneal T cells at an inductive phase of macrophage activation in our system.

Previous observations pointed out that the most cytotoxic macrophages were the largest cells [7, 13, 14]. Chapes and Haskill reported that for macrophages activated by *C. parvum*, only those containing ingested *C. parvum* were cytotoxic [7]. Our data also showed that the larger cell fractions (C, D, and E) were prominent in cytotoxic activity. It is of interest to investigate whether there is a correlation between intracellular SPG content and cytostatic activity. Ultrastructural analysis will be required to elucidate the relationship between the number of SPG-containing peritoneal macrophages and their tumor suppressive activities. Such an approach is currently in progress.

We also found that SPG had no mitogenic effects on murine B and T cells (Table 6). This is interesting because well-known macrophage stimulants such as *C. parvum*, BCG and muramyl dipeptide (MDP) are lymphocyte mitogens [4, 17]. SPG-treated peritoneal and bone-marrow-derived macrophages affected the cell growth of various cell lines, and cell-to-cell contact seemed to be important for effective tumor cell toxicity, although there are contradictory data for this [26]. However, we still cannot say that there is little possibility that SPG-treated macrophages secrete tumor cell toxic factor(s) and kill tumor cells nonspecifically without cellular contact, because cytotoxicity may take place in a serum-free condition without cellular contact [1].

Suzuki et al. reported that SPG-treated cells involved in the anti-tumor activity were shown to be T cells since anti-tumor activity was diminished when lymph-node cells obtained from SPG-treated mice were treated with anti-Thy-1.2 sera and complement, and that macrophages were also shown to be involved since administration of carrageenan (CGN) or trypan blue into the host decreased the inhibition rate of tumor cell growth after IM injection of SPG in tumor-bearing mice [31]. As experimental systems differ, it is difficult to compare our data with theirs. However, at least we can say that peritoneal macrophages are playing a role in anti-tumor activity.

It is also conjectured that in Suzuki's experimental system SPG-treated T cells secrete lymphokine, which renders macrophages cytotoxic, and cytotoxic macrophages may play a major role in tumor cell cytotoxicity. In our in vitro system

macrophages activated directly with SPG seemed to suppress tumor cell growth (Table 4).

Tumor rejection by normal and nude mice following *C. parvum* administration in vivo suggests that T-independent and T-dependent activation of macrophages seem to exist [32]. Considering our data further (Tables 3–5), it is suggested that there may exist T-independent and T-dependent mechanisms of macrophage activation induced by SPG.

In conclusion, the most cytotoxic macrophages are the largest mature cells, and SPG seems to activate peritoneal macrophages, as identified morphologically, in a direct fashion to render them cytotoxic. We think that our observation may turn out to be useful for further progress in immunotherapy since tumoricidal macrophages can be identified ultrastructurally and our system will allow us to investigate further roles of SPG, whose molecular structure is well-known, in the direct activation of macrophages and inhibition of cancer metastasis.

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